

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|-------------|--|-----------------|--------------------|
| Applicant: | Mitani et al. | Examiner: | Mummert, Stephanie |
| Serial No.: | 10/583706 | Group Art Unit: | 1637 |
| Filed: | April 12, 2007 | Docket No.: | 20078.0001USWO |
| Title: | METHOD OF AMPLIFYING NUCLEIC ACID AND METHOD OF DETECTING MUTATED NUCLEIC ACID USING THE SAME | | |

DECLARATION UNDER 37 CFR §1.132

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Yasumasa Mitani, hereby declare as follows:

1. I graduated from
1994, March; School of Medicine, Faculty of Medicine, Tokushima University.
1996, March; Master's Course, Graduate School of Nutrition and Bioscience, Tokushima University.
2009, January ; Doctor's degree (Nutritional science)

2. I have worked in
1996, April; Wakunaga Pharmaceutical Co.,Ltd.
2006, April; Kabushiki Kaisha DNAFORM.

3. I consider myself to be an expert in the field of molecular biology.

4. Under my direction, the following experiments were conducted.

Experiment

1. Experiment Objective

Using FP + TP (present invention) and PCR primer + TP (comparative test) as primer pairs, the amplification effects of these primer pairs were compared.

2. Experiment Description

2. 1. Amplification Experiment

In this example, using Human Genomic DNA (manufactured by Promega) as a template, it was attempted to amplify the human STS DYS237 gene contained therein. As primers, a primer pair 1 or a primer pair 2 having the following sequences was used. Furthermore, the positional relationship of each primer region to the template was set as illustrated in FIG. 1. A forward primer F1 is designed so as to have the structure shown in FIG. 2 in which the sequence (22 mer: the underlined part) that is located on its 3' end side anneals to the template, while the sequence (16 mer: the part other than the underlined part) that is located on the 5' end side is folded in that region. A forward primer F2 is designed so as to be used for common PCR that the sequence (22 mer: the underlined part) that is located on its 3' end side anneals to the template. A reverse primer R1 is designed so that the sequence (20 mer: the double-underlined part) that is located on its 3' end side anneals to the template, while after an extension reaction, the sequence (10 mer: the part other than the double-underlined part) that is located on the 5' end side hybridizes to the region starting from 16 bases downstream of residues located at the 3' end of the primer on the strand extended by the primer.

The primer pairs used in this experiment are as follows.

Primer pair 1 (FP + TP)

F1: 5'- ggatatatatatccactgaacaaatgccacataaag -3' (FP)

R1: 5'- gcagcatcaccaacccaaaagcactgagta -3' (TP)

Primer pair 2 (PCR primer + TP)

F2: 5'- actgaacaaatgccacataaag -3' (PCR primer)

R1: 5'- gcagcatcaccaacccaaaagcactgagta -3' (TP)

A reaction solution (25 μ L) having the following composition was prepared: Tris-HCl (20 mM, pH 8.8), KCl (10 mM), $(\text{NH}_4)_2\text{SO}_4$ (10 mM), MgSO_4 (8 mM), DMSO (3%), Triton X-100 (1%), dNTP (1.4 mM), 2000 nM of each primer of the aforementioned primer pairs, a template (100 ng), and 16U Bst DNA polymerase (NEW ENGLAND BioLabs). This was incubated at 60°C for 1 hour. The template was allowed to react while being maintained in the double-stranded state. The same experiment was carried out with respect to a solution in which sterile water was added instead of the template.

With respect to each reaction solution 2 μ L, electrophoresis was carried out at 100 V for 60 minutes using 4% NuSieve 3:1 Agarose (manufactured by BioWhittaker Molecular Applications (BMA) Inc.; purchased from TAKARA BIO INC.). After the electrophoresis, the gel thus obtained was stained with ethidium bromide (EtBr) and thereby nucleic acids

were detected. The results are shown in Lanes 1 to 12 in FIG. 3. The samples in the respective lanes shown in FIG. 3 are as follows:

Lane 1: 20 bp DNA Ladder size marker (manufactured by Fermentas)

Lanes 2 and 3: reaction solutions each using the primer pair 1 and containing a template

Lanes 4 and 5: reaction solutions each using the primer pair 2 and containing a template

Lanes 6 and 7: reaction solutions each using the primer pair 1 and to which sterile water instead of a template was added

Lanes 8 and 9: reaction solutions each using the primer pair 2 and to which sterile water instead of a template was added

Lane 10: 20 bp DNA Ladder size marker (manufactured by Fermentas)

Lanes 11 and 12: reaction solutions obtained by digesting solutions of Lanes 2 and 3 with a restriction enzyme

In Lanes 2 and 3 shown in FIG. 3, among the small size bands, the bands located around 120 bp are expected as amplification products of the target nucleic acid. Thus, an amplification product was observed in the reaction solution containing the template. In Lanes 2 and 3, further, bands were observed above the amplification product. They are amplification products that contain the target nucleic acid sequence repeatedly, which are predicted in the amplification reaction according to the present invention. The amplification products obtained through the amplification reaction according to the present invention have complicated structures. As a result, such a ladder-like electrophoresis result was obtained. In Lanes 4 to 9, no bands were observed other than those of stained unreacted primers (especially, FPs in Lanes 6 and 7).

2. 2. Confirmatory Experiment of Amplification Product with Restriction Enzyme

In order to prove that the amplification products obtained in 5. 1. were derived from the target nucleic acid sequence, the amplification products were digested with a restriction enzyme. Specifically, 0.3 μ L of each of the amplification reaction solutions of Lanes 2 and 3 obtained after the amplification reaction in 5. 1. was digested (at 37°C for 3 hours) with a restriction enzyme MboII. The digestion product was electrophoresed using 4% NuSieve 3:1 Agarose (manufactured by BioWhittaker Molecular Applications (BMA) Inc.; purchased from TAKARA BIO INC.). The results are shown in Lanes 11 and 12 in FIG. 3. The samples in the respective lanes shown in FIG. 3 are as described above.

In FIG 3, the size of each fragment digested with the restriction enzyme that is predicted from the nucleotide sequence is indicated on the right-hand side of the electropherogram. Since the bands of the undigested sample were changed into the bands

with predicted sizes (about 110 bp and about 75 bp) after the digestion, it was proved that the target nucleic acid sequence had been amplified.

I declare under the penalty of perjury of the laws of the United States of America that the foregoing is true and correct to the best of my information and belief.

Signed this 6 of Feb, 2012, at Yokohama, JAPAN

Yasumasa Mitani

Yasumasa MITANI